

TERMINAL LABELED PROBE ARRAY AND METHOD  
OF MAKING IT

BACKGROUND OF THE INVENTION

5 Field of the Invention

[0001]

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The present invention relates to a labeled probe  
array, a method for producing the labeled probe array,  
and an method for determining the amount of a target  
10 substance using the labeled probe array.

Related Background Art

[0002]

Recently, for precise assessment of a target  
substance, it has been intensively studied a method  
15 using a plurality of probes disposed in an array on the  
surface of a substrate, called "probe array". A  
typical example is a so-called "DNA probe array" in  
which a plurality of DNA probes are disposed on a  
substrate. This DNA probe array is also referred to as  
20 a DNA chip. By using the DNA probe array, for example,  
the presence of a plurality of target genes can be  
analyzed at the level of base sequence.

[0003]

Methods of making the probe array are roughly  
25 classified into two.

One is a method that synthesizes probes by  
sequential synthesis on a solid substrate, applicable

to the probes being a substance that can be synthesized by chemical elongation and of which nucleotide or amino acid sequence has an important meaning, e.g., DNA, oligonucleotide, peptide nucleic acids (PNAs), proteins, and oligopeptides.

[0004]

As an example of such a method, U.S. Patent No. 5,445,966 discloses a method of synthesizing a DNA probe array on a substrate by using photolytic protection groups and photolithography. Alternatively, U.S. Patent No. 5,474,796 discloses a method supplying nucleotide monomers by an ink jet process.

[0005]

The other method is applicable, in addition to the above described substances, to the substances not chemically synthesizable, since this method is to supply probes that have been previously synthesized or prepared to the matrix sites of a probe-array substrate by the micro-dispensing method, pin transfer method, or ink jet process etc.

[0006]

Since the second method uses probes which have been purified and of which quantity can be controlled, the purity of the probes are secured and the concentrations of the probes to be reacted with the substrate can be freely determined. Consequently, the final amounts of respective probes on the substrate

surface can be assessed with a certain accuracy, to enhance the reliability of the target substance detection using the probe array. On the other hand, the second method requires previous synthesis of all kinds of probes for the probe array and it also requires to apply the probes to the substrate for reaction. Sometimes, several hundreds of thousands of probes may be required for a probe array. In that case, it will be considerably difficult to synthesize all probes beforehand.

[0007]

On the other hand, according to the first method, necessary probes are synthesized on the substrate and then used for the reaction with the target substances, so that operations for immobilizing the previously synthesized or prepared probes onto the substrate can be omitted.

[0008]

However, in order to improve the detection sensitivity or reliability of the first method, following points must be improved.

(1) The yield in each step of the sequential synthesis is not 100%, some times the final yield is as low as 5%. This directly leads to reduction in the sensitivity when the target substance is detected.

(2) For the same reason, both full length probes and shorter length probes are present at each matrix

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site of the probe array. This lowers the reliability in detecting a target substance.

(3) As a result of in situ synthesis on the substrate, the amount of the probe at each matrix site cannot be measured. This also lowers the reliability in detecting a target substance.

(4) As mentioned above, the synthetic yield in each step of sequential synthesis is not 100% and the synthetic yield varies depending on the steps and the matrix sites, so that the final amounts of respective probes vary broadly. This also lowers the reliability of the detection of a target substance.

#### SUMMARY OF THE INVENTION

[0009]

The object of the present invention is to solve the above described problems.

[0010]

According to the present invention, there is provided a method of making a probe array for capturing a target substance which comprises the steps of:

synthesizing a plurality of probes immobilized at a plurality of matrix sites on a substrate by sequential synthesis where constitutional units are added one by one to a plurality of basal parts immobilized to the substrate until a desired length is obtained; and

coupling a labeling compound to a terminus of the probe of the desired length.

[0011]

Further, according to the present invention, there  
5 is provided a probe array which comprises a plurality  
of probes immobilized at a plurality of matrix sites on  
a substrate for capturing a target substance, wherein  
the probes are sequentially synthesized at the matrix  
sites on the substrate until a desired length, the  
10 probes are different from each other, and a labeling  
compound is coupled to each terminus of the probes in a  
final step of the synthesis.

[0012]

The present invention also provides a method of  
15 measuring an amount of a probe in a probe array wherein  
the probe array comprises a plurality of probes  
immobilized at a plurality of matrix sites on a  
substrate for capturing a target substance, the probes  
are sequentially synthesized at the matrix sites on the  
20 substrate until a desired length, the probes are  
different from each other, and a labeling compound is  
coupled to each terminus of the probes in a final step  
of the synthesis, which method comprises the step of  
measuring an amount of the labeling compound at each  
25 matrix site.

[0013]

The present invention provides a method of

evaluating an amount of a target substance, which comprises the steps of:

reacting a probe array and a target substance wherein the probe array comprises a plurality of probes immobilized at a plurality of matrix sites on a substrate for capturing a target substance, the probes are sequentially synthesized at the matrix sites on the substrate until a desired length, the probes are different from each other, and a labeling compound is coupled to each terminus of the probes in a final step of the synthesis;

measuring an amount of the labeling compound at each matrix site to determine an amount of the probe at the matrix site;

measuring an amount of a labeled target substance captured by the probe at the matrix site; and

comparing the amount of the probe with the amount of the labeled target substance.

[0014]

The present invention also provides a method of evaluating an amount of a target substance comprising the steps of:

reacting a probe array and a target substance, wherein the probe array comprises a plurality of probes immobilized at a plurality of matrix sites on a substrate for capturing a target substance, the probes are sequentially synthesized at the matrix sites on the

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5           measuring an amount of the labeling compound at  
each matrix site to determine an amount of the probe at  
the matrix site;

10            measuring an amount of the labeling compound  
directly bonded to the substrate at a predetermined  
matrix site on the surface of the substrate during a  
first step of the sequential synthesis without  
elongation reaction; and

### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows an probe arrangement on the array substrate with nucleotide sequences of a target DNA and probes;

FIG. 3 shows the structural formula of a target DNA;

FIG. 5 shows corrected result of the hybridization of Example 3;

FIG. 7 shows a comparison of the result of FIG. 6 with the result of FIG. 5.

10 DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0016]

The feature of the present invention is to introduce a quantitatively determinable labeling substance to the probe in the final step of the sequential synthesis.

[ 0017 ]

The sequential synthesis is a method for synthesizing a concatenate oligomer consisting of from several to around one hundred units (for example, DNA consisting of nucleotides and protein consisting of amino acid residues) by sequentially bonding the desired units. This conventional method widely used in synthesis of oligonucleotides, oligopeptides, and PNAs can be used in the present invention without any alterations thereto. In case of DNA (oligonucleotide) synthesis, for example, the most commonly used method with an automatic synthesizer at present is the



phosphoramidite method. This method comprises the following steps of:

(1) deprotecting hydroxyl groups bonded to the surface of a solid carrier (a glass substrate etc.) via a

5 linker;

(2) coupling the deprotected hydroxyl group to the 3'phosphorous group of an amidite monomer having a desired base;

(3) capping the hydroxyl groups not reacted in step(2);

10 (4) oxidizing the coupled amidite in step (3) from phosphite to phosphate;

(5) removing the protection group protecting 5' hydroxyl moiety of the coupled amidite in step (2);

15 (6) repeating steps (2) to (5) to obtain an oligonucleotide of a desired length and base sequence in a direction from 3' to 5'; and

(7) deprotecting the nucleotide bases.

[0018]

Examples of the probes are, but not limited to,  
20 RNAs, DNAs, oligonucleotides, peptide nucleic acids (PNAs), proteins, oligopeptides or the like.

[0019]

In detecting a target substance using the probe array, it is common to label the target substance with  
25 a fluorescent dye. Therefore, it is convenient to label the probes with a fluorescent dye, because the labeling substance can be detected by the same measuring device

[ 0020 ]

[ 0021 ]

15 [0022]

[ 0023 ]

Therefore, in the present invention, only probes that have elongated to the final step (to a desired chain length) are labeled with a labeling substance, so that the measurement of the amount of the labeling substance at each matrix site allows evaluation of the

probe quantity at each matrix site.

[0024]

Thus, relative correction between respective probes in determining the amount of the target substance can be done by comparing the quantitatively determined probe amount and the amount of the labeling substance of the target substance captured by the probe.

[0025]

10 In order to determine the absolute amount of the captured target substance in the present invention, a control site is provided on the substrate where only the first hydroxyl groups introduced to the substrate are labeled with a labeling substance without  
15 subsequent elongation reaction steps, and the amount of the labeling substance at the control site is compared with the respective amounts of the labeling substance coupled to the probes synthesized at the matrix sites by elongation reaction. This allows to evaluate  
20 relative amounts of the synthesized probes to the initial functional groups introduced to the substrate surface. By comparing thus determined amount of the probe in each matrix with the amount of the labeling substance coupled to the target substance that was  
25 captured by the probe, evaluation of the absolute amount of the target substance and relative correction between the probes can be carried out simultaneously.

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[0026]

Any procedures disclosed in the above described U.S. Patents and other conventional techniques can be used for respective steps in preparing the terminal-  
5 labeled probe array of the present invention.

[0027]

The present invention will now be particularly described by the following Examples.

Example 1 Making of a terminal labeled DNA probe array

10 [0028]

(1) A synthetic quartz substrate (25.4 mm × 25.5 mm × 0.5 mm) was subjected to ultrasonication for 20 minutes with a 10% aqueous solution of a ultrasonic detergent (GP-II, a product of Branson Co.), washed  
15 with ultra pure water, dipped in an aqueous solution of 1 N sodium hydroxide (80°C), washed with ultra pure water, dried, and then subjected to the UV ozone treatment to clean the surface thereof.

[0029]

20 (2) Next, DEEP-UV resist containing carbon black (BK-739P, a negative photoresist for black matrix, Nippon Steel Chemical Co., Ltd.) was applied to the substrate to a thickness of 5 μm by using a spin coater. This substrate was heated at 80°C for 5  
25 minutes on a hot plate to pre-bake the resist. This resist film was subjected to proximity exposure by using a DEEP-UV aligner and a pattern mask

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corresponding to the matrix pattern where the matrix sites of  $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$  are arranged at  $100\text{ }\mu\text{m}$  intervals within an area of  $1\text{ cm} \times 1\text{ cm}$  as shown in FIG. 1. Then the resist was developed with an

5 inorganic alkaline aqueous solution in a spin developer, and rinsed with ultra pure water to completely remove the inorganic alkaline aqueous solution. This substrate was roughly dried with the use of a spin dryer, then heated to  $180^{\circ}\text{C}$  for 30  
10 minutes in a clean oven in order to post-bake the resist. Consequently, 2500 matrix sites, each of which being surrounded with the resist film, were formed on the surface of the substrate. The volume of each matrix site thus formed is calculated as 50 pL.

15 [0030]

(3) An aqueous solution of 1% silane coupling agent having epoxy groups (KBM403:  $\gamma$ -glycidoxypopyltrimethoxysilane, Shin-Etsu Chemical Co., Ltd.) was hydrolyzed by stirring it for one hour  
20 at room temperature. The substrate provided with the above described matrix sites thereon was dipped in this solution for one hour and cleaned with ultra pure water properly, then heated at  $120^{\circ}\text{C}$  for one hour. As a result, epoxy groups were bonded to the above described  
25 matrix sites. By treating the substrate with hexaethylene glycol, hydroxyl groups were introduced to the matrix sites for coupling oligonucleotides.

[0031]

(4) The synthesis of oligonucleotides on the substrate was carried out by the ordinary phosphoramidite method. A polyethylene chamber to contain the above described substrate therein was fabricated and then mounted in a column part of a DNA synthesizer (381A, a product of ABI Inc.). Synthesizing steps other than substance application of amidite monomers and activators, subsequent cleaning, and drying steps were carried out by the DNA synthesizer. A modified piezo ink-jet system was employed in supplying the amidite monomers and activators. Operating the piezo ink-jet apparatus in a dry box (argon), not inks but acetonitrile solutions of four amidite monomers and an activator were applied to the above described matrix sites on the substrate under the position control. The liquid amount supplied at a time from the piezo device was 24 pL on average.

[0032]

(5) Basically, it is possible to synthesize distinct oligonucleotides for all of 2500 matrix sites. In this example, however, 64 oligonucleotides, i.e., DNA probes, were synthesized. These 64 oligonucleotides include every base variation at three positions in two triplets encoding two amino acids in a carcinogenic gene p53. The DNA probe array substrate obtained was dipped in a 30% aqueous solution of ammonia for 12

hours at room temperature to deprotect the nucleic acid bases.

[ 0033 ]

Base sequences of synthesized DNA probes (SEQ ID NO: 1 to SEQ ID NO: 64) are as follows.

[illegible]

- No.1. 5'-GATGGGACTCAAGTTTCAT-3'  
No.2. 5'-GATGGGACTCAGGTTTCAT-3'  
No.3. 5'-GATGGGACTCACGTTTCAT-3'  
No.4. 5'-GATGGGACTCATGTTTCAT-3'  
5 No.5. 5'-GATGGGACTCGAGTTTCAT-3'  
No.6. 5'-GATGGGACTCGGGTTTCAT-3'  
No.7. 5'-GATGGGACTCGCGTTTCAT-3'  
No.8. 5'-GATGGGACTCGTGTTTCAT-3'  
No.9. 5'-GATGGGACTCCAGTTTCAT-3'  
10 No.10. 5'-GATGGGACTCCGGTTTCAT-3'  
No.11. 5'-GATGGGACTCCCGTTTCAT-3'  
No.12. 5'-GATGGGACTCCTGTTTCAT-3'  
No.13. 5'-GATGGGACTCTAGTTTCAT-3'  
No.14. 5'-GATGGGACTCTGGTTTCAT-3'  
15 No.15. 5'-GATGGGACTCTCGTTTCAT-3'  
No.16. 5'-GATGGGACTCTTGTTTCAT-3'  
No.17. 5'-GATGGGGCTCAAGTTTCAT-3'  
No.18. 5'-GATGGGGCTCAGGTTTCAT-3'  
No.19. 5'-GATGGGGCTCACGTTTCAT-3'  
20 No.20. 5'-GATGGGGCTCATGTTTCAT-3'  
No.21. 5'-GATGGGGCTCGAGTTTCAT-3'  
No.22. 5'-GATGGGGCTCGGGTTTCAT-3'  
No.23. 5'-GATGGGGCTCGCGTTTCAT-3'  
No.24. 5'-GATGGGGCTCGTGTTTCAT-3'  
25 No.25. 5'-GATGGGGCTCCAGTTTCAT-3'  
No.26. 5'-GATGGGGCTCCGGTTTCAT-3'  
No.27. 5'-GATGGGGCTCCCGTTTCAT-3'

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- No. 55. 5'-GATGGGGTCTCGCGTTTCAT-3'  
No. 56. 5'-GATGGGGTCTCGTGTTTCAT-3'  
No. 57. 5'-GATGGGGTCTCCAGTTTCAT-3'  
No. 58. 5'-GATGGGGTCTCCGGTTTCAT-3'  
5 No. 59. 5'-GATGGGGTCTCCCGTTTCAT-3'  
No. 60. 5'-GATGGGGTCTCCTGTTTCAT-3'  
No. 61. 5'-GATGGGGTCTCTAGTTTCAT-3'  
No. 62. 5'-GATGGGGTCTCTGGTTTCAT-3'  
No. 63. 5'-GATGGGGTCTCTCGTTTCAT-3'  
10 No. 64. 5'-GATGGGGTCTCTTGTTTCAT-3'

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[0034]

FIG. 1 shows base sequences of DNA probes on the DNA array substrate and an arrangement thereof as well as base sequences of tetramethyl rhodamine labeled model target DNA to be used for subsequent hybridization. The sequence No. 65 (SEQ ID NO: 65) is a target DNA sequence and complementary probe sequences No. 1 to 64 differ each other at three underlined base positions, where one of A, G, C and T comes to create  $4^3$  = 64 combinations. The three base positions of the above described probes correspond to the underlined parts in the sequence No. 65. The position denoted by N in each sequence corresponds to A, G, C and T written above the upper edge of the box. As a result, DNA probe No.42 is completely complementary to the target DNA, and others have one, two, or three mismatches within the sequences as illustrated in FIG. 1.

[0035]

(6) A fluorescent dye, fluorescein, was bound to the 5'-terminal of every synthesized DNA probe by using fluorescein phosphoramidite (Glenn Research Co.). In addition, fluorescein was directly bound to another matrix site using fluorescein amidite, at the matrix site, fluorescein was directly coupled with the hydroxy groups of the substrate using fluorescein phosphoramidite instead of amidite in step (2). In this case, elongation reaction will not proceed in principle

in subsequent steps.

Example 2 Quantitative fluorometry of the DNA probe array synthesized in Example 1.

[0036]

5 Fluorescence of the fluorescein in each matrix site was taken in by using a fluorescence microscope ECLIPSE800 (Nikon Corp., an objective lens CFIPlanApo 20x) and a CCD camera C2400-87 equipped with an image intensifier (Hamamatsu Photonics K.K.), then analyzed  
10 by an image processing device, Argu50 (Hamamatsu Photonix Co., Ltd.). An amplification degree HV of the image intensifier was 2.0, integration was performed 64 times, and B-2E/C for the fluorescein measurement was employed as a filter block of the fluorescence  
15 microscope. In the measurement of the fluorescence, a substrate was placed on a slide glass and 50 mmol/L of a phosphate buffer (pH = 7.0, containing 100 mmol/L NaCl) was dropped onto the substrate appropriately, then a cover slip was put thereon for the measurement.

20 [0037]

FIG. 2 shows measured fluorescence values. The fluorescence value (11330) written outside the square in FIG. 2 was from a matrix site where the fluorescein was directly bound to the substrate. FIG. 2 shows that  
25 fluorescence values obtained from the bonded oligonucleotides range from 2940 to 10350. These values can be used to correct the amounts of respective

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oligonucleotide probes, and the hybridization results can be corrected by using these fluorescence values. When a plurality of DNA arrays are used, it is possible to correct the values of the matrix sites using the fluorescence value of the control matrix site where fluorescein has been directly bonded to the substrate in respective DNA arrays.

Example 3 Assay of a target DNA using the DNA probe array synthesized in Example 1.

10 [0038]

FIG. 3 shows a structure of a tetramethyl rhodamine-labeled model target DNA used for hybridization. This target DNA sequence corresponds to the sequence No. 65 as mentioned above.

15 [0039]

The above described probe array was dipped in a 50 mmol/L phosphate buffer (pH = 7.0, containing 100 mmol/L of NaCl and 2% bovine serum albumin (Sigma-Aldrich Japan) for one hour, then rinsed with the above described buffer appropriately, and subjected to hybridization.

[0040]

The hybridization was carried as follows:

First the DNA probe array and 2 mL of a 50 mmol/L phosphate buffer (pH = 7.0, 100 mmol/L NaCl) containing the above described target DNA at a concentration of 50 nmol/L were enclosed in a resin pack for hybridization,

and heated to 70°C and then cooled to 20°C, and left standing for 24 hours.

[0041]

5       Next, the substrate was rinsed in the above buffer  
for 20 minutes, then fluorescence from tetramethyl  
rhodamine of the target DNA was measured in the same  
manner as in Example 2. In this case, Y-2E/C was  
employed as a fluorescence filter block. In addition,  
the amplification degree of the image intensifier was  
10       4.0.

[0042]

15       The result is shown in FIG. 4. FIG. 5 shows  
values after correction obtained by multiplying the  
measured values of FIG. 4 by a correction coefficient (  
control fluorescence value from fluorescein directly  
bonded to the substrate without elongation reaction  
11330/measured fluorescence from fluorescein at the  
corresponding matrix site where elongation reaction had  
been carried out). These values are compared with  
20       values obtained from Comparative Examples (as described  
later).

Comparative Example 1   Application of synthesized DNA  
probes to a substrate by ink-jet process, probe  
coupling and hybridization

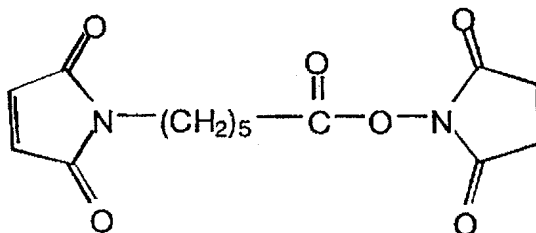
25       [0043]

A glass substrate was cleaned as described in  
Example 1. An 1% aqueous solution of aminosilane

coupling agent (KBM-603, Shin-Etsu Chemical Co., Ltd.)  
purified by vacuum distillation was stirred for one  
hour at room temperature to hydrolyze the methoxy  
moiety thereof. The above treated substrate was rinsed  
5 and immediately dipped in the above described aqueous  
solution of silane coupling agent for one hour at room  
temperature. Then the substrate was rinsed with running  
water (ultra pure water), dried by blowing nitrogen  
gas, and then fixed in an oven for one hour at 120°C.

10 [0044]

After cooling, the substrate was dipped in a 0.3%  
solution (ethanol:dimethylsulphoxide = 1:1) of N-(6-  
maleimidocaproxy)succinimide (EMCS, Dojindo Co.)  
(Compound I below) for two hours at room temperature  
15 for reaction, and then it was rinsed with a solution of  
ethanol:dimethylsulphoxide = 1:1 once and rinsed with  
ethanol three times, and dried by blowing nitrogen gas.



Compound I

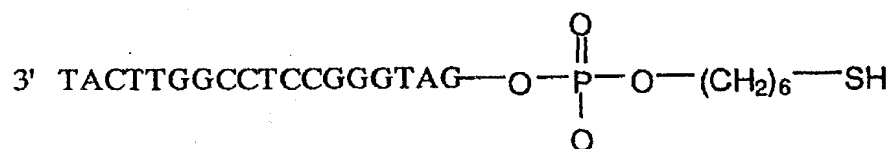
[0045]

This treatment coupled the amino group of the  
25 silane coupling agent on the glass substrate and the  
succinimide group of EMCS, so that a maleimide group was  
formed on the substrate surface. This maleimide group

is coupled to the thiol group of a DNA probe (as described later).

[0046]

Sixty four kinds of DNA probes having completely the same sequences as described in Example 3 were purchased from Becks K.K. Each of these DNAs carries a thiol linker at 5'-terminal thereof for coupling to the substrate. An example of the DNA having the thiol linker is illustrated as Compound II below.



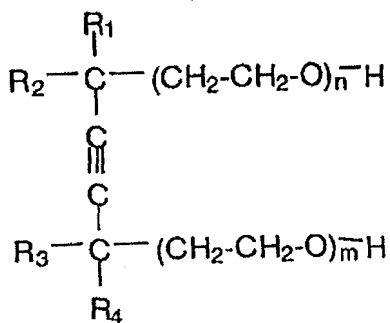
Compound II

This compound II has a base sequence (No. 42) which is completely complementary to the model target DNA.

[0047]

Each DNA probe was dissolved in a solvent to be ejected from a thermal jet printer, that is, each DNA probe was dissolved in an aqueous solution containing 7.5 wt% of glycerin, 7.5 wt% of urea, 7.5 wt% of thiodiglycol, and 1 wt% of acetylene alcohol represented by the following general formula III (trade name: Acetylenol EH, Kawaken Fine Chemicals Co., Ltd.) to an absorbance of 1.0.





Compound III

[0048]

Then 0.4 mL of each solution was charged in a modified ink supplying part of a thermal jet printer, which printer can hold two ink-jet heads (Canon Inc.). Then the solutions were discharged onto the above described substrate to form the same pattern as in Examples 1 to 3 with different shapes. According to the configuration of this apparatus, the amount of one drop of the discharged solution was 24 pL, and the dot diameter formed by one drop of the solution became 70 to 100 μm under these conditions. The discharging density was 120 dpi (dot/inch). After probe application, the substrate was placed in a moisture chamber at 100% humidity for one hour at room temperature for reaction, then rinsed with running water (ultra pure water) for 30 seconds.

[0049]

Treatment with BSA, conditions for hybridization, and conditions of quantitative fluorometry were the same as described in Example 3. Amplification degree

of the image intensifier was 2.0. The result of quantitative fluorometry is shown in FIG. 6.

[0050]

FIG. 7 shows the comparison of the results of FIG. 5 and the results of FIG. 6. The former shows the corrected hybridization results obtained by using an array of DNA probes sequentially synthesized and terminal labeled, corrected by the fluorescence value of the fluorescent dye used for labeling the probe terminus and the fluorescent value of the fluorescent dye directly bonded to the substrate, and the latter shows the hybridization results obtained by using a DNA probe array where the synthesized and purified probes were bonded to the substrate.

[0051]

Value  $C_n$  in the matrix of FIG. 7 was calculated by the following equation (1), where  $A_n$  is a value from each matrix site of FIG. 5 and  $B_n$  is a value from each matrix site of FIG. 6 ( $n = 1$  to 64).

$$(1) \quad C_n = B_n \times (\Sigma A_n / \Sigma B_n) / A_n$$

( $\Sigma A_n$  represents a sum of  $A_1$  to  $A_{64}$  and  $\Sigma B_n$  represents a sum of  $B_1$  to  $B_{64}$ .)

[0052]

FIG. 7 shows the effectiveness of the correction of the present invention. Values indicated in FIG. 7 are within a range of  $\pm 20\%$  and the standard deviation is within a range of about 9%. It is also possible to

5 [0053]

As described above, according to the present invention, one can evaluate the probe amount at each matrix site by terminal labeling of the sequentially synthesized probes. Also it enables to detect and  
10 quantify the target substance by correcting respective probe amounts. Further, direct coupling of a labeling substance to the substrate enables correction between probe arrays.